

Supporting Information

Synthesis and Preliminary Evaluation of *N*-(16-

[¹⁸F]fluorohexadecanoyl)ethanolamine (¹⁸F-FHEA) as a PET Probe of

***N*-Acylethanolamine Metabolism in Mouse Brain**

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Description of Figures:

Figure S1: IH-NMR of N-(16 fluorohexadecanoyl)ethanolamine (**3**)

Figure S2: 19F-NMR of N-(16 fluorohexadecanoyl)ethanolamine (**3**)

Figure S3: Flow schematic of the TRACERlab FXN Pro with microwave reactor insertion

Scheme SS1. Synthesis of nonradioactive standards

Figure S4: Radio TLC of ¹⁸F-(CH₂)₁₅CONHCH₂CH₂OTHP crude product (¹⁸F-analog of compound 8, scheme S-I)

Figure S5: Radio-TLC of ¹⁸F-(CH₂)₁₅CONHCH₂CH₂OH crude product (compound 4, scheme I main manuscript)

Figure S6: MicroPET images of ¹⁸F-FHEA with and without NAAA inhibitor 25-30 min p.i.

S-2

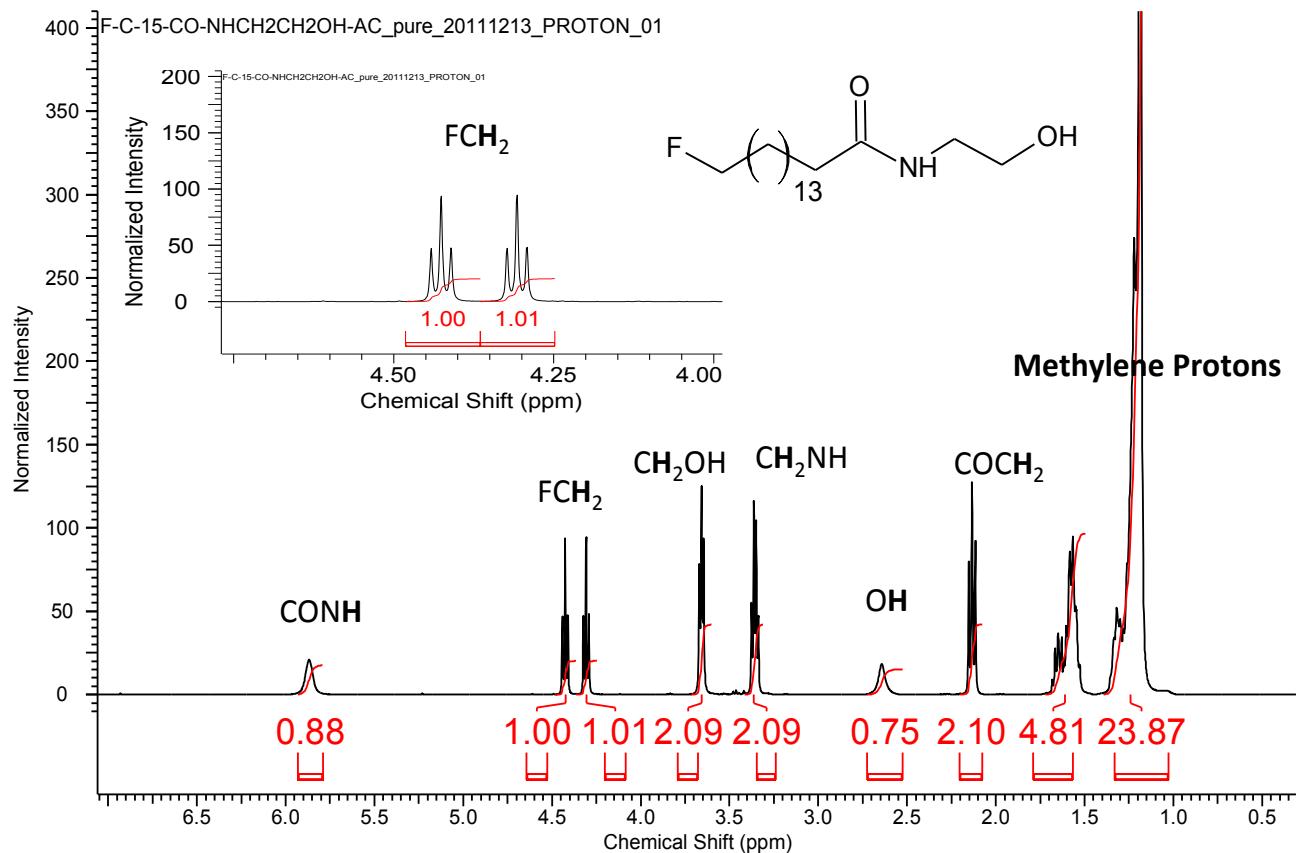


Figure S1: ¹H-NMR of ¹H- NMR characterization of *N*-(16 fluorohexadecanoyl)ethanolamine (3)

(3)

S-3

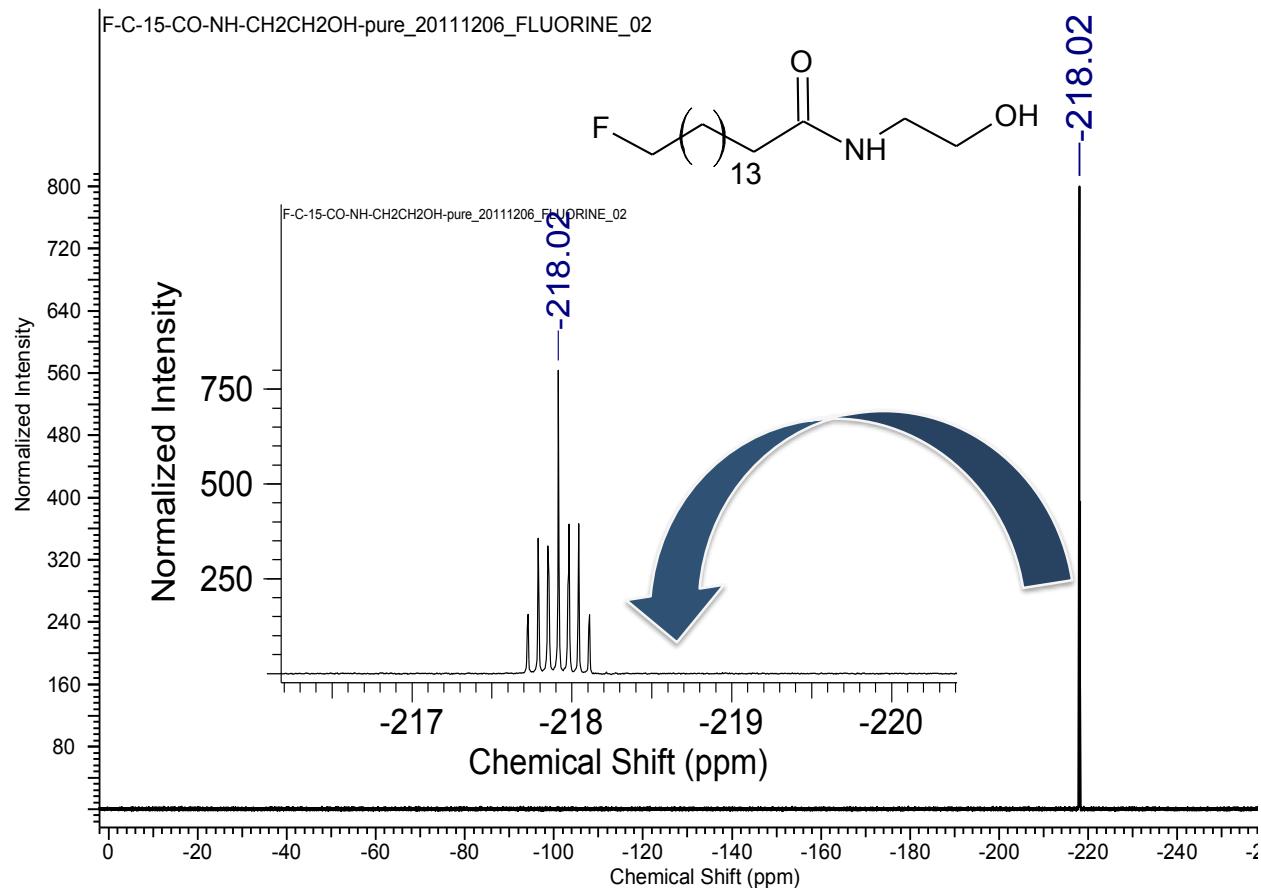
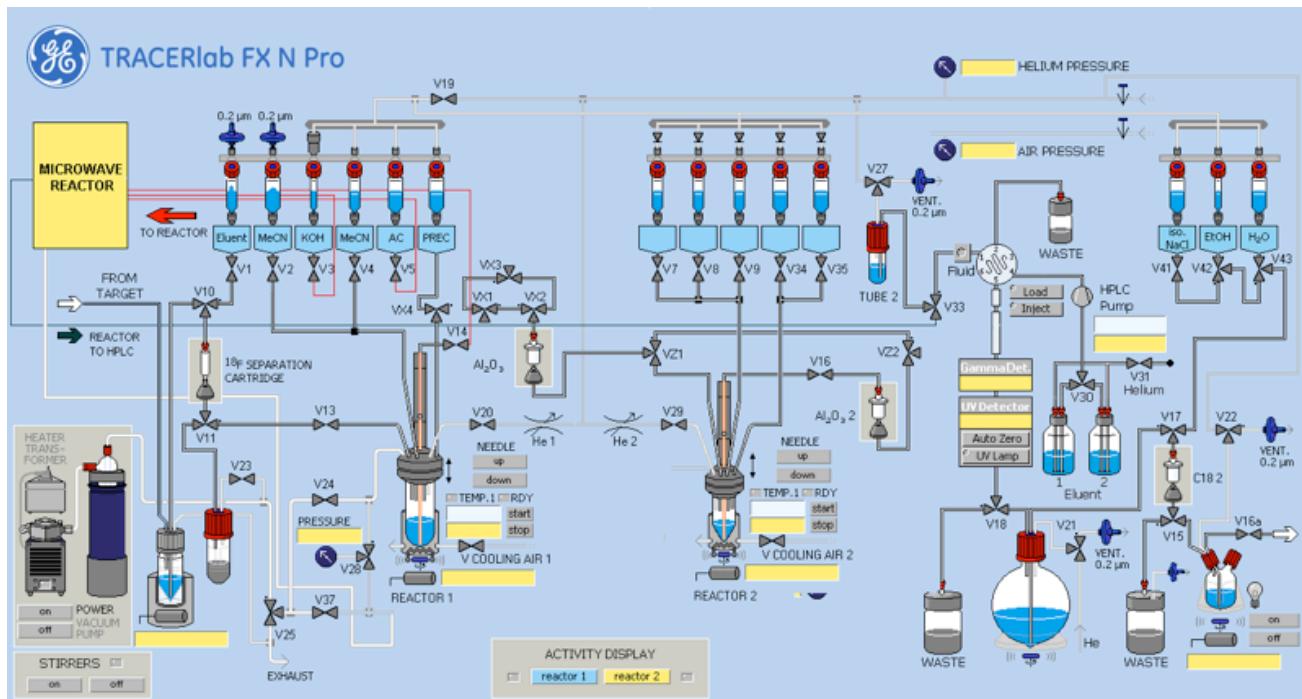
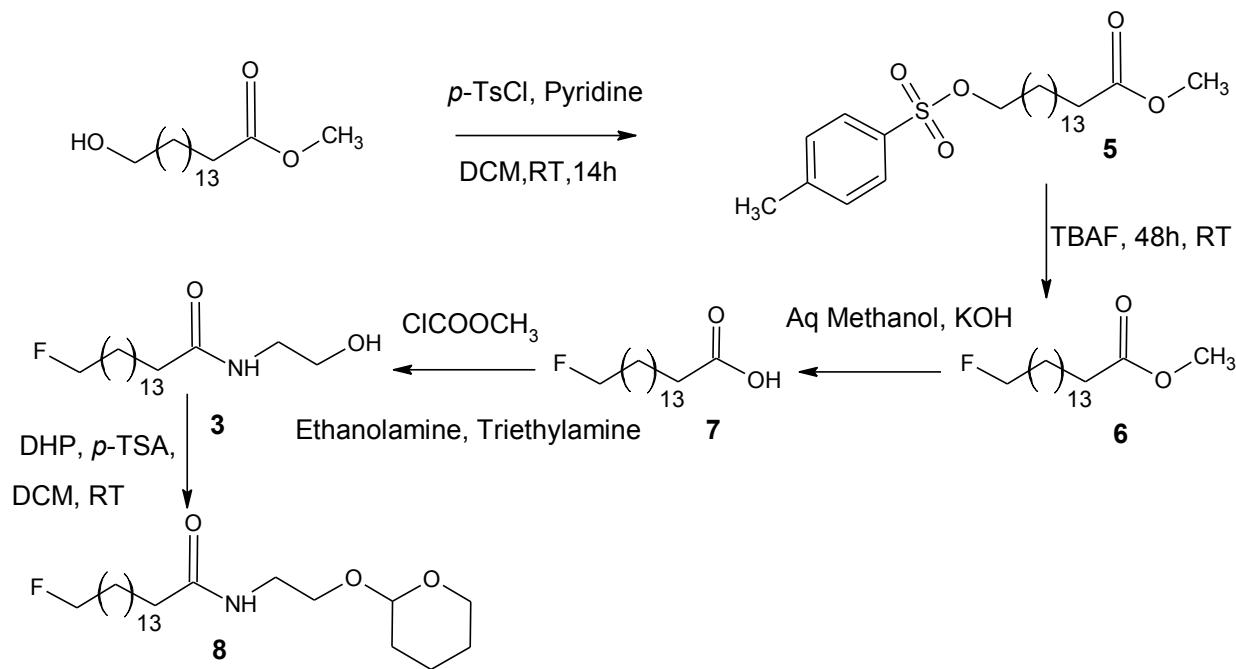


Figure S2: ¹⁹F-NMR of *N*-(16 fluorohexadecanoyl)ethanolamine (**3**)



Scheme SS1: Synthesis of nonradioactive standards**Synthesis of methyl 16-p-tosyloxyhexadecanoate (5)**

Synthesis of methyl 16-p-tosyloxyhexadecanoate (**5**) was achieved by stirring of methyl 16-hydroxyhexadecanoate (3.00 g, 10.48 mmol), pyridine (1.659 g, 20.9 mmol) and *p*-toluenesulfonyl chloride (2.60 g, 13.63 mmol) in anhydrous dichloromethane at room temperature for 12 h. After completion, reaction mixture was diluted with diethyl ether and extracted against water and brine solution. Organic layer was dried over anhydrous sodium sulfate, filtered and the obtained filtrate was concentrated under reduced pressure. The desired compound was purified by column chromatography using 05:95 ethyl acetate: petroleum ether as eluting solvent to yield compound **5** (4.10 g, 89 % yield, Mp 50±2 °C (Lit.²⁴ Mp 54-55 °C) as a white solid.

¹H NMR (25 °C, 599.77 MHz, CDCl₃) δ ppm: 7.73 (d, 2H, *J* = 12.0 Hz), 7.28 (d, 2H, *J* = 6.0 Hz), 3.95 (t, 2H, *J*_{1,2} = 6.0 Hz -OCH₂-), 3.60 (s, 3H, -OCH₃), 2.38 (s, 3H, -CH₃), 2.23 (t, 2H, *J*_{1,2} = 6.0 Hz, CH₂), 1.56 (m, 2H, CH₂), 1.22-1.14 (brs, 24H, 12xCH₂). ¹³C NMR (25 °C, 150.81 MHz, CDCl₃) δ ppm: 177.0 (-COOCH₃), 147.2, 135.9 (CH_x2), 132.4 (CH_x2), 130.5, 73.3 (-OCH₂), 54.0 (-OCH₃), 36.7 (CH₃), 32.2-31.4 (CH₂x11), 27.9, 27.6, 24.2. HRMS (ES) calcd for C₂₄H₄₀O₅S (M⁺) 440.25964, found 440.26035.

Synthesis of methyl 16-fluorohexadecanoate (6)

Synthesis of methyl 16-fluorohexadecanoate (**6**) was achieved by stirring an anhydrous acetonitrile solution of methyl 16-*p*-tosyloxyhexadecanoate (**5**) (3.00 g, 6.8 mmol) and 1M THF solution of TBAF (3 ml) at room temperature for 72 h under nitrogen. The additional, 0.8 ml 1M THF solution was further added at 24 h and 48 h. After completion, solvent was evaporated under vacuum and the resulting solid was dissolved in 100 mL of dichloromethane, washed with water and brine solution, dried over anhydrous Na₂SO₄ and filtered. The filtrate was evaporated under vacuum to dryness. The obtained fluoro-ester residue was further subjected to column chromatography using 25:75 chloroform: petroleum ether as solvent and silica gel as an adsorbent to obtain compound **6** as a white low meting solid (1.5 g, 76 % overall yield). ¹H NMR (25 °C, 599.77 MHz, CDCl₃) δ ppm: 4.40 (dt, 2H, *J*₁ = 42.0 Hz, *J*₂ = 6.0 Hz), 3.60 (s, 3H, -OCH₃), 2.23 (t, 2H, *J*_{1,2} = 6.0 Hz), 1.65-1.60 (m, 2H), 1.56-1.53 (m, 2H), 1.33-1.29 (m, 2H), 1.22-1.19 (brs, 20H, 10xCH₂). ¹³C NMR (25 °C, 150.81 MHz, CDCl₃) δ ppm: 176.9 (COOCH₃), 87.4 and 86.3 (d, *J* = 165.8 Hz, FCH₂-), 54.0 (-OCH₃), 36.7, 33.1, 33.0, 32.2-32.0 (CH₂x7),

31.9, 31.8, 27.8-27.6 (d, J = 30.1 Hz), 27.6. ^{19}F NMR (25 °C, 376.02 MHz, CDCl_3) δ ppm: - 218 (m, 1F, CH_2F). HRMS (ES) calcd for $\text{C}_{17}\text{H}_{33}\text{O}_2\text{F}$ (M^+) 288.2464, found 288.2472.

Synthesis of 16-fluorohexadecanoic acid (7)

Synthesis of 16-fluorohexadecanoic acid (7) was achieved by stirring methyl 16-fluorohexadecanoate (**6**) (1.00 g, 3.47 mmol) in methanol and 0.5 mL aqueous solution of KOH (0.194 g, 3.47 mmol) at room temperature for 2 h. The solvent was evaporated under vacuum. The obtained residue was dissolved in 25 ml of dichloromethane, washed with 1N hydrochloric acid, dried over Na_2SO_4 and filtered. The filtrate was evaporated under vacuum and purified by column chromatography using 5:95 methanol: chloroform as solvent and silica gel as an adsorbent to obtain compound **7** as a white solid (0.80 g, 84 % overall yield, Mp 79±2 °C).

^1H NMR (25 °C, 599.77 MHz, CDCl_3) δ ppm: 4.41 (dt, 2H, J_1 = 48.0 Hz, J_2 = 6.0 Hz), 2.28 (t, 2H, $J_{1,2}$ = 6.0 Hz), 1.64-1.60 (m, 2H), 1.58-1.54 (m, 2H), 1.33-1.30 (m, 2H), 1.22-1.19 (brs, 20H, 10x CH_2). ^{13}C NMR (25 °C, 100.48 MHz, CDCl_3) δ ppm: 178.6 (COOH), 85.0 and 83.4 (d, J = 160.7 Hz, FCH_2 -), 33.7, 30.5, 30.3, 29.6-29.0 (CH_2 x9), 25.2-25.0 (d, J = 20 Hz), 24.6. ^{19}F NMR (25 °C, 376.02 MHz, CDCl_3) δ ppm: - 218 (m, 1F, CH_2F). HRMS (ES) calcd for $\text{C}_{16}\text{H}_{31}\text{O}_2\text{F}$ (M^+) 274.2315, found 274.2308.

Synthesis of *N*-(16-fluorohexadecanoyl)ethanolamine (3) starting from 16-fluorohexadecanoic acid (7)

Synthesis of *N*-(16-fluorohexadecanoyl)ethanolamine (3) was also achieved in good yield starting from 16-fluorohexadecanoic acid (7) (0.50 g, 1.82 mmol), and stirring with methyl chloroformate (0.344 g, 3.64 mmol), triethylamine (0.368 g, 3.64 mmol) in dichloromethane at 0 °C for 30 min and then at room temperature for an additional hour and half. After 2h of stirring, reaction temperature was further lowered to 0 °C to add ethanolamine (0.222 g, 3.64 mmol). The resultant reaction mixture was stirred overnight at room temperature. After completion, solvent was removed under vacuum and cold water was poured in to the flask containing residue with constant stirring for additional 10 min. Solid obtained was filtered and dried. TLC was re-examined for desired product in 8:92 methanol: chloroform as a solvent system. The residue was subjected to column chromatography using silica gel as an adsorbent and 5:95 methanol: chloroform as solvent to yield compound 1 (0.485 g, 84 % yield, Mp 97±1 °C) as a white solid. (for detailed characterization see compound 3)

Synthesis of 16-fluoro-*N*-[2[(tetrahydro-2*H*-pyran-2-yl)oxy]ethyl]hexadecanoylamide (8)

Synthesis of 16-fluoro-*N*-[2[(tetrahydro-2*H*-pyran-2-yl)oxy]ethyl]hexadecanoylamide (8) was achieved by stirring of *N*-(16-fluorohexadecanoyl)ethanolamine (3) (0.40 g, 1.26 mmol), 3,4-dihydro-2*H*-pyran (0.137 g, 1.64 mmol) in dichloromethane using *p*-toluenesulfonic acid as catalyst (0.260 g, 1.51 mmol). The mixture was stirred for 24 h at room temperature and quenched with water. After extraction with the chloroform, organic layer was dried over Na₂SO₄ and solvent was evaporated under vacuum. The residue was subjected to

chromatography (2:98 methanol: chloroform) to yield **8** (1.30 g, 79% yield, Mp 53±1 °C) as a white solid. ¹H NMR (25 °C, 599.77 MHz, CDCl₃) ppm: 5.95 (brs, 1H, NH), 4.48 (t, 1H), 4.42-4.32 (dt, 2H, *J*₁ = 48.0 Hz, *J*₂ = 6.0 Hz, CH₂F), 3.81 (m, 1H), 3.69 (m, 1H), 3.54 (m, 1H), 3.45 (m, 1H), 3.39 (m, 2H, CH₂NH), 2.11 (t, 2H, *J*_{1,2} = 6.0 Hz, CH₂), 1.76 (m, 2H, CH₂), 1.62 (m, 2H, CH₂), 1.53 (m, 2H, CH₂), 1.41 (m, 2H, CH₂), 1.32 (m, 2H, CH₂), 1.22-1.18 (brs, 22H, 11xCH₂). ¹³C NMR (25 °C, 100.48 MHz, CDCl₃) δ ppm: 173.1 (CONH), 99.8 (OCHO), 85.0 and 83.4 (d, *J* = 160.7 Hz, FCH₂-), 67.1, 63.2, 39.4, 36.8, 30.7, 30.5, 30.3, 29.6-29.2 (CH₂x9), 25.7, 25.3, 25.2-25.0 (d, *J* = 20 Hz), 20.0. ¹⁹F NMR (25 °C, 376.02 MHz, CDCl₃) δ ppm: -218 (m, 1F, CH₂F). HRMS (ES) calcd for C₂₃H₄₄O₃NF (M⁺) 401.3305, found 401.3311.

S-10

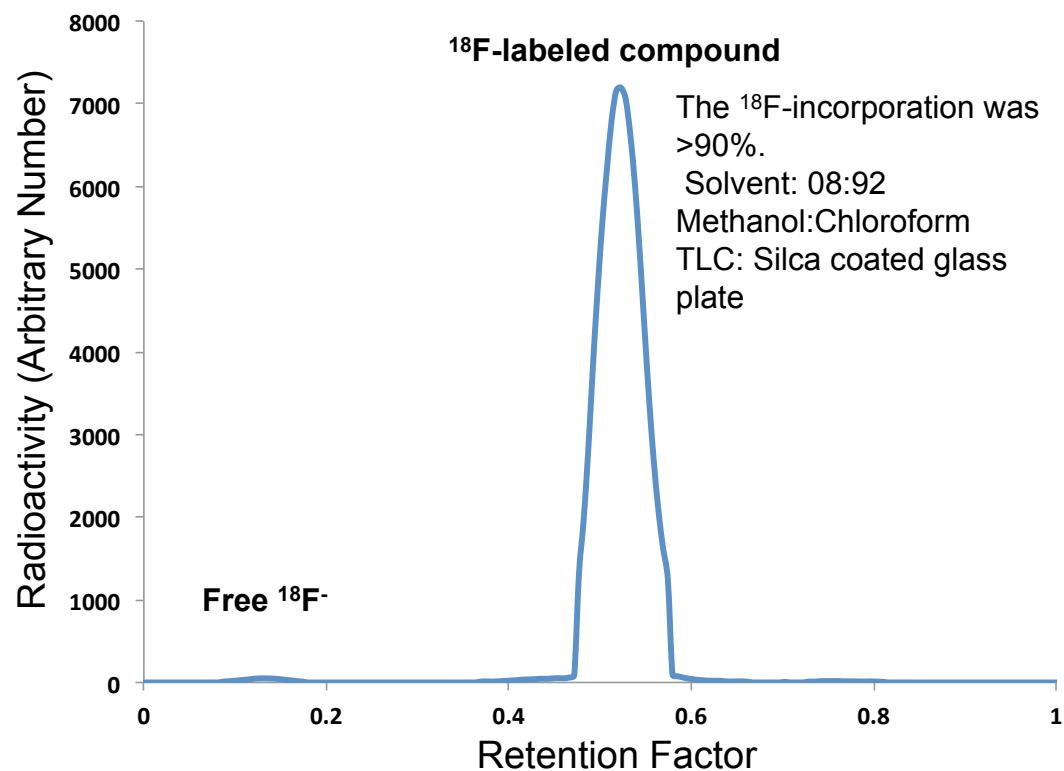


Figure S4: Radio-TLC of $^{18}\text{F}-(\text{CH}_2)_{15}\text{CONHCH}_2\text{CH}_2\text{OTHP}$ crude product (^{18}F -analog of compound 8, scheme SS1)

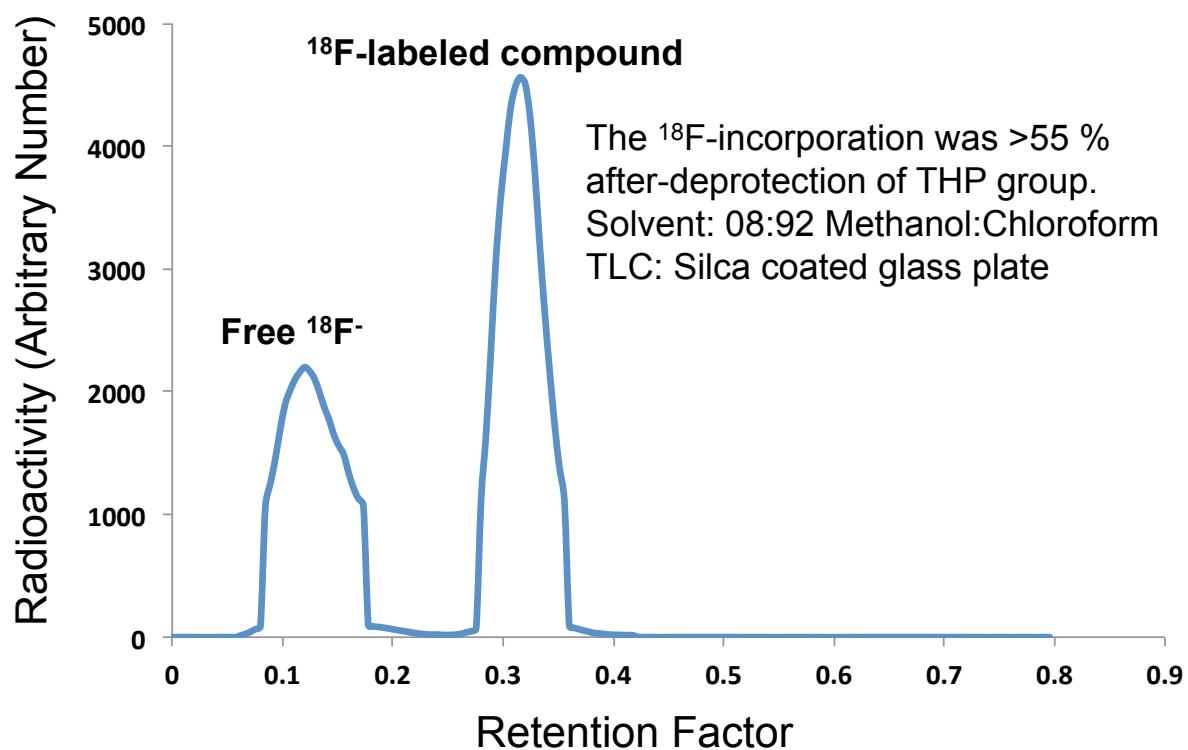


Figure S5: Radio-TLC of $^{18}\text{F}-(\text{CH}_2)_{15}\text{CONHCH}_2\text{CH}_2\text{OH}$ crude product (Compound 4, Scheme I, main manuscript)

PET imaging

PET imaging of [¹⁸F]FHEA was investigated in Swiss Webster mice under the approval of the Mayo Clinic Institutional Animal Care and Use Committee. The animals were anesthetized with isoflurane (2-3%) before injection of the radiotracer and remain anesthetized throughout the study isoflurane (1.5-2%). Mice were pretreated 15 min prior with 20 mg/kg of NAAA inhibitor (Compound 7h) or vehicle injected into a tail vein. Dynamic PET imaging was performed over 30 m using Sofie BioSystems Genesys4 PET scanner following retro-orbital bolus administration of [¹⁸F]FHEA (~1 MBq). Following the PET emission acquisition, PET images were reconstructed by an iterative OSEM algorithm.

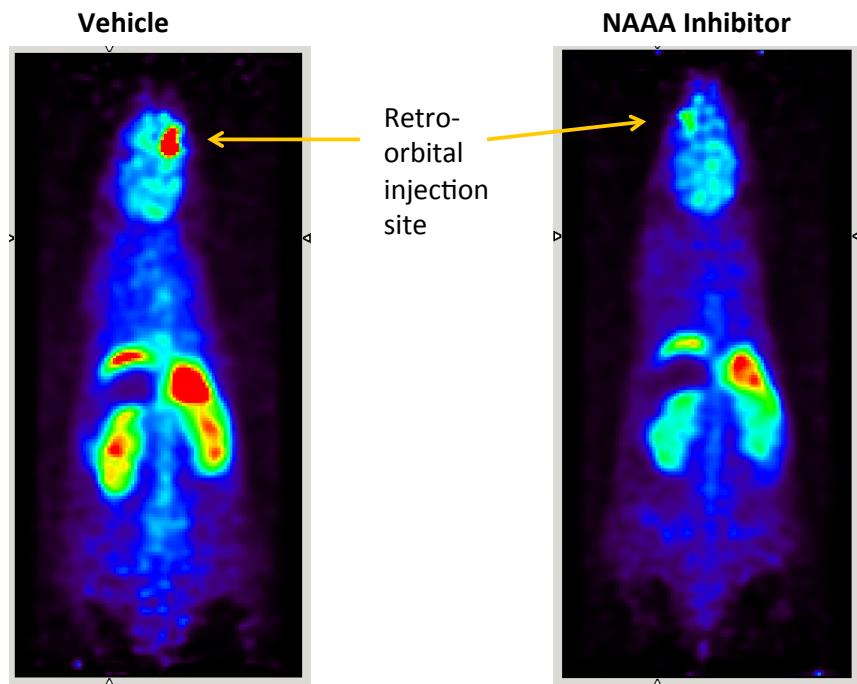


Figure S6: MicroPET images of ^{18}F -FHEA with and without NAAA inhibitor 25-30 min p.i.